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WITNESS my hand this
Eighth day of December 2003

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Regulation 3.2

Murdoch Childrens Research Institute

AUSTRALIA

Patents Act 1990

PROVISIONAL SPECIFICATION

for the invention entitled:

"A diagnostic assay"

The invention is described in the following statement:

A DIAGNOSTIC ASSAY

BACKGROUND OF THE INVENTION

FIELD OF THE INVENTION

The present invention relates generally to a method for genotyping a subject to identify a likelihood of that subject developing a pathological condition. More particularly, the present invention provides genotyping of deafness or an associated disorder using hybridization of single-stranded test DNA to a sequence-specific oligonucleotide. Even more particularly, the present invention employs microarray analysis to identify the presence of heterozygous or homozygous wild-type or mutant sequences of a gene or other nucleic acid target. This provides the genotype of a particular gene or nucleic acid target. The present invention may be provided in kit form and may be conducted manually, automatically or semi-automatically. The identification of a subject's genotype with respect to a gene or other target nucleic acid facilitates corrective therapy at the medical or behavorial level.

DESCRIPTION OF THE PRIOR ART

Bibliographic details of references provided in the subject specification are listed at the end of the specification.

Reference to any prior art in this specification is not, and should not be taken as, an acknowledgment or any form of suggestion that this prior art forms part of the common general knowledge in any country.

Deafness is one of the most common human genetic conditions. Approximately one child in 1000 is born with a prelingual hearing loss which will have a significant impact on the infant's speech, language and general development, incurring lifelong social, educational and economic costs (Yoshinaga, *Otolaryngol Clin. North Am. 32(6):* 1089-1102, 199?).

Approximately 10% of the population are affected by age-related hearing loss by the age of 60 years and 50% by the age of 80 years (Davis, Hearing in adults, London: Whurr, 1995). More than half of prelingual deafness has a genetic basis and defects in many genes, probably more than 100, can cause deafness. More than 20 genes have been identified to date (Petit *et al., Annu. Rev. Genet. 35:* 589-646, 2001). Despite this genetic heterogeneity, a small group of genes are known to account for the majority of genetic non-syndromic hearing loss. For example, mutations in the *connexin 26* gene are responsible for over half of autosomal recessive non-syndromic hearing loss. Mutations in the *pendrin* gene can cause both non-syndromic and syndromic (Pendred Syndrome) deafness and are estimated to cause up to 10% of genetic hearing loss. The A1555G mitochondrial 12S rRNA mutation has been reported at a high frequency in Spanish and Japanese families with severe progressive deafness and can induce hearing loss upon exposure to aminoglycosides, which are commonly given in high doses to premature babies. Mutations in the *usherin* gene are largely responsible for the most common form of Usher Syndrome, type II, which is characterized by congenital deafness with onset of retinitis pigmentosa in late teens (Van Camp and Smith, Hereditary hearing loss homepage, URL: http://dnalab-www.uia.ac.be/dnalab/hhh/).

The genetic heterogeneity of deafness has proved a challenge for genetic testing: analysis of multiple genes by conventional gel-based methods is both time-consuming and expensive. There is a need, therefore, to develop more efficient and accurate means of identifying mutations or polymorphisms in genes and nucleic acid molecules associated with genetic deafness.

SUMMARY OF THE INVENTION

Throughout this specification, unless the context requires otherwise, the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated element or integer or group of elements or integers but not the exclusion of any other element or integer or group of elements or integers.

Nucleotide and amino acid sequences are referred to by a sequence identifier number (SEQ ID NO:). The SEQ ID NOs: correspond numerically to the sequence identifiers <400>1 (SEQ ID NO:1), <400>2 (SEQ ID NO:2), etc. A summary of the sequence identifiers is provided in Table 1. A sequence listing is provided at the end of the specification.

The present invention is directed to a sequence-specific oligonucleotide-based genotyping of one or more target genes or target nucleic acid molecules in a single subject or in multiple subjects. More particularly, the present invention employs sequence-specific oligonucleotides directed to particular alleles or mutations or polymorphisms in genes or other nucleic acid molecules (e.g. rRNA) associated with a pathological condition such as deafness. Genetic deafness is heterogenous and there are more than 60 linked loci and more than 20 genes associated with this condition. The present invention combines microarray technology with sequence specific oligonucleotide hybridization to screen for one or a multiplicity of genes in a single subject or in a number of subjects. The sequence-specific oligonucleotide is also referred to herein as an allele-specific oligonucleotide.

The nucleic acid microarray, or biochip, is a new hybridization-based genotyping technique that offers simultaneous analysis of many genetic mutations. The parallelism offered by the microarray platform makes it ideally suited to genotyping of genetically heterogeneous conditions such as deafness.

Allele-specific oligonucleotides to genes or other target nucleic acid molecule such as *connexin 26, pendrin,* mitochondrial 12S rRNA and *usherin* are immobilized onto a solid support. The solid support is preferably planar such as on a microchip or biochip.

However, the present invention is also applicable on spheres and nanoparticles, each coded by a reporter molecule or other characteristic feature. DNA from a subject to be tested is amplified and labeled with a reporter molecule and rendered single-stranded before being brought into contact with the immobilized allele-specific oligonucleotides.

Alternatively, the presence or absence of a test DNA which has hybridized to an immobilized sequence specific oligonucleotide may be achieved by hybridizing a labeled oligonucleotide (referred to as a reporter oligonucleotide) to, for example, a particular nucleotide sequence on the target DNA distinct from the nucleotide sequence which encompasses the mutation. Conveniently, a nucleotide tail of, for example, Ts or As may be used as a generic tag for a reporter oligonucleotide.

Still in a further alternative, the label may be a nucleotide capable of creating a current. Such nucleotides are referred to as an electrotide. Such technology uses the complementary binding properties of DNA and RNA to create an electric circuit.

Hybridization or non-hybridization is determined by the presence or absence of the signal of the reporter molecule. An algorithm is then used to define the genotype index (GI), wherein:

$$GI = \frac{SV_N}{SV_N + SV_M}$$

wherein:

SV_N is the normal spot value; and

SV_M is the mutant spot value.

The value is the level of signal of the reporter molecule. Preferably, the reporter molecule is a fluorescent molecule including a fluorophore.

The present invention provides, therefore, a method for genotyping a subject with respect to a gene or target nucleic acid sequence associated with a pathological condition, said method comprising contacting an allele specific oligonucleotide immobilized to a solid support with a single-stranded form of DNA from a subject to be tested labeled directly or indirectly with a reporter molecule capable of giving an identifiable signal under conditions which permit hybridization of single stranded DNA which is exactly complementary to the immobilized allele specific oligonucleotide but substantially less or no hybridization of non-complementary single-stranded DNA molecules and then screening for the presence or absence or level of reporter molecule which provides an indicator of the genetic identity of the single-stranded DNA molecule which in turn provides the genotype of the subject.

Examples of preferred oligonucleotides are shown in Table 1. The oligonucleotides may have a sequence of particular nucleotides or of a single type of nucleotide at the immobilization end of the molecule. This is the case for SEQ ID NOs:1 to 32 which have $[T]_x$ where x is 10. Alternatively, a chemical linker may be used between the solid support and the oligonucleotide. Furthermore, the target sequence may be modified using mismatched primers to interrupt sequences of particular nucleotides which my otherwise adversely affect hybridization.

A summary of the allele specific oligonucleotides and corresponding SEQ ID NOs is shown in Table 1 for each gene tested.

TABLE 1

List of allele specific oligonucleotides and genes used to detect genotype

GENE	MUTATION	OLIGONUCLEOTIDE SEQUENCE	SEQ ID NO:
Connexin 26			
35delG	35W18A	TTTTTTTTTTGATCCTGGGGGGTGTGAA	1
	35W18B	TTTTTTTTTTATCCTGGGGGGTGTGAAC	2
	35M17A	TTTTTTTTTTGATCCTGGGGGTGTGAA	3
	35M17B	TTTTTTTTTTATCCTGGGGGTGTGAAC	4
M34T	34W16	TTTTTTTTTTCGCATTATGATCCTCG	5
	34M16	TTTTTTTTTTCGCATTACGATCCTCG	6
167delT	167W18	TTTTTTTTTTCAACACCCTGCAGCCAGG	7
	167M17	TTTTTTTTTTCAACACCCGCAGCCAGG	8
235delC	235W16	TTTTTTTTTTTTATGGGCCCTGCAGCT	9
	235M15	TTTTTTTTTTTTATGGGCCTGCAGCT	10
V37I	37W16	TTTTTTTTTTTGATCCTCGTTGTGGCT	11
	37M18	TTTTTTTTTTTGATCCTCATTGTGGCTG	12
W24X	24W19	TTTTTTTTTTGAAAGATCTGGCTCACCGT	13
	24M19	TTTTTTTTTTGAAAGATCTAGCTCACCGT	14
L90P	90W19	TTTTTTTTTTCAGCGCTCCTAGTGGCCAT	15
	90W17	TTTTTTTTTTAGCGCTCCCAGTGGCCA	16
R143W	143W17	TTTTTTTTTTTCTTCTTCCGGGTCATC	17
	143M19	TTTTTTTTTTATCTTCTTCTGGGTCATCT	18
313del14	313W20	TTTTTTTTTTAGGAAGTTCATCAAGGGGGA	19
	313M20	TTTTTTTTTTGAAGAAGAGGGGAGATAAAG	20
Pendrin			
L236P	236W21	TTTTTTTTTTGTCTCACAGCTAAAGATTGTC	21
	236M21	TTTTTTTTTTGTCTCACAGCCAAAGATTGTC	22
1001	1001W17	TTTTTTTTTTCCAAGGGGGTGAGTGTG	23
	1001M18	TTTTTTTTTTCCAAGGGGATGAGTGTGG	24
E384G	384W21	TTTTTTTTTGCTTCCTTAGGAATTCATTGCC	25
	384M19	TTTTTTTTTTTTCCTTAGGGATTCATTGC	26
T416P	416W19	TTTTTTTTTTCAGGAGAGCACTGGAGGAA	27
	416M19	TTTTTTTTTTCAGGAGAGCCCTGGAGGAA	28
Mitochondrial rRNA			
A1555G	1555W21	TTTTTTTTTTTTATAGAGGAGACAAGTCGTAA	29

GENE	MUTATION	OLIGONUCLEOTIDE SEQUENCE	SEQ ID NO:
	1555M21	TTTTTTTTTTTTATAGAGGAGGCAAGTCGTAA	30
Usherin			
2299delG	2299W20	TTTTTTTTTTGGGCAGTGTGAGTGCAAAAA	31
	2299M17	TTTTTTTTTTGGCAGTGTAGTGCAAAA	32
Connexin 26			
35delG	35W18A	GATCCTGGGGGGTGTGAA	33
	35W18B	ATCCTGGGGGGTGTGAAC	34
	35M17A	GATCCTGGGGGTGTGAA	35
	35M17B	ATCCTGGGGGTGTGAAC	36
M34T	34W16	CGCATTATGATCCTCG	37
	34M16	CGCATTACGATCCTCG	38
167delT	167W18	CAACACCCTGCAGCCAGG	39
	167M17	CAACACCCGCAGCCAGG	40
235delC	235W16	ATGGGCCCTGCAGCT	41
	235M15	ATGGGCCTGCAGCT	42
V37I	37W16	GATCCTCGTTGTGGCT	43
	37M18	GATCCTCATTGTGGCTG	44
W24X	24W19	GAAAGATCTGGCTCACCGT	45
	24M19	GAAAGATCTAGCTCACCGT	46
L90P	90W19	CAGCGCTCCTAGTGGCCAT	47
	90W17	AGCGCTCCCAGTGGCCA	48
R143W	143W17	CTTCTTCCGGGTCATC	49
	143M19	ATCTTCTTCTGGGTCATCT	50
313del14	313W20	AGGAAGTTCATCAAGGGGGA	51
	313M20	GAAGAAGAGGGGAGATAAAG	52
Pendrin			
L236P	236W21	GTCTCACAGCTAAAGATTGTC	53
	236M21	GTCTCACAGCCAAAGATTGTC	54
1001	1001W17	CCAAGGGGGTGAGTGTG	55
	1001M18	CCAAGGGGATGAGTGTGG	56
E384G	384W21	GCTTCCTTAGGAATTCATTGCC	57
	384M19	CCTTAGGGATTCATTGC	58
T416P	416W19	CAGGAGAGCACTGGAGGAA	59
	416M19	CAGGAGAGCCCTGGAGGAA	60
Mitochondrial rRNA			
A1555G	1555W21	ATAGAGGAGACAAGTCGTAA	61
	1555M21	ATAGAGGAGGCAAGTCGTAA	62
Usherin			
2299delG	2299W20	GGGCAGTGTGAGTGCAAAAA	63
	2299M17	GGCAGTGTAGTGCAAAA	64

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 is a diagrammatic representation showing attachment chemistry for allele-specific oligonucleotides to microarray solid support.

Figure 2 is a diagrammatic representation showing microarray based genotyping using allele-specific oligonucleotides.

Figure 3 is a photographic representation showing genotyping of *connexin 26* 35ΔG and M34T mutations.

Figure 4 is a graphical representation of the genotype index (GI) of *connexin 26* 35ΔG and M34T mutations.

Figure 5 is a photographic representation of genotyping of *connexin 26* mutations 35ΔG/M3RT, 35DG/35ΔG, M34T/M34T, 167delT/N, 167delT/167delT, 235delC/N and V371/N. N = normal; M = mutant.

Figure 6 is a photographic representation of genotyping of pending and 12S rRNA mutations. *Pendrin*: 1001 G > A, E384G, T416P and L236P. 12SrRNA: A1555G. N = normal; M = mutant.

Figures 7(a)-(n) are graphical representations showing the genotype index (GI) of various genes associated with deafness.

Figure 8 is a graphical representation of genotype algorithms to determine N/N (homozgyous normal), N/M (heterozygos normal) and M/M (homozygous mutant).

Figure 9 is a graphical and tabular representation showing interactions between deafness genes.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention provides a method for genotyping a particular subject with respect to a gene or other target nucleic acid molecule such as an mRNA or rRNA. More particularly, the present invention combines allele (i.e. sequence) specific oligonucleotide hybridization specificity with microarray analysis in order to genotype a subject with respect to a gene or genes or other target nucleic acid molecules associated with a pathological condition.

Accordingly, one aspect of the present invention contemplates a method for genotyping a subject with respect to a gene or target nucleic acid sequence associated with a pathological condition, said method comprising contacting an allele specific oligonucleotide immobilized to a solid support with a single-stranded form of DNA from a subject to be tested labeled directly or indirectly with a reporter molecule capable of giving an identifiable signal under conditions which permit hybridization of single stranded DNA which is exactly complementary to the immobilized allele specific oligonucleotide but substantially less or no hybridization of non-complementary single-stranded DNA molecules and then screening for the presence or absence or level of reporter molecule which provides an indicator of the genetic identity of the single-stranded DNA molecule which in turn provides the genotype of the subject.

Reference to direct or indirect labeling includes incorporating a label or a labeled nucleotide into the test DNA during PCR or alternatively using labeled oligonucleotides which hybridize to portions of the test DNA not associated with a mutation. For example, a sequence of nucleotides such as As, T, Gs or Cs or mixtures thereof may be added to a target DNA. A labeled oligonucleotide sequence complementary to the introduced nucleotide sequence is then used to determine the presence or absence of an immobilized target DNA sequence.

A label includes a reporter molecule capable of giving an identifiable signal (e.g. a fluorescent molecule) or a nucleotide capable of creating an electrical current or other electrical signal.

The present invention applies to a range of pathological conditions within a range of subjects. Such subjects include humans, primates, livestock animals, laboratory test animals, companion animals and captured wild animals. However, the present invention is particularly directed to identifying genotypes associated with genetic deafness or a propensity for development of genetic deafness in human subjects.

The present invention is hereinafter described in relation to this preferred embodiment. This is done, however, with the understanding that the subject invention extends to a range of pathological conditions including *inter alia* autoimmune diseases, inflammatory conditions, cancer, neurological disorders and neurodegenerative disorders.

Accordingly, in a preferred embodiment, the present invention provides a method for genotyping a human with respect to a gene or target nucleic acid sequence associated with genetic deafness, said method comprising contacting an allele specific oligonucleotide immobilized to a solid support with a single-stranded form of DNA from a human to be tested labeled directly or indirectly with a reporter molecule capable of giving an identifiable signal under conditions which permit hybridization of single stranded DNA which is exactly complementary to the immobilized allele specific oligonucleotide but substantially less or no hybridization of non-complementary single-stranded DNA molecules and then screening for the presence or absence or level of reporter molecule which provides an indicator of the genetic identity of the single-stranded DNA molecule which in turn provides the genotype of the human.

The allele specific oligonucleotides are designed to differentially hybridize to a target nucleotide sequence based on at least one nucleotide difference. For example, a polymorphism or mutation at a single or multiple nucleotide positions may occur in genes in subjects suffering from genetic deafness or having a propensity to suffer from this disorder. An allele specific oligonucleotide is designed to either hybridize to a "mutant" form of a nucleotide sequence or to a "wild-type" form of the sequence. The term "allele-specific oligonucleotide" may also be read as "sequence-specific oligonucleotide". The

term "allele" is not to impart any limitation.

The immobilized allele (i.e. sequence) specific oligonucleotides may target different polymorphisms or mutations within a single gene or may target polymorphisms or mutations in multiple genes (i.e. two or more genes). Furthermore, the allele specific oligonucleotides may cover the same or multiple mutations in two or more subjects. Consequently, the allele specific oligonucleotides are in effect an array of nucleic acid molecules which exhibit complementarity to a nucleotide sequence from a healthy subject not exhibiting genetic deafness or a nucleotide sequence from a subject exhibiting genetic deafness.

Accordingly, another aspect of the present invention contemplates a method for genotyping a subject with respect to one or a multiplicity of genes or target nucleic acid associated with genetic deafness, said method comprising contacting an array of allele specific oligonucleotides immobilized to a solid support with a single-stranded form of DNA from a subject to be tested labeled directly or indirectly with a reporter molecule capable of giving an identifiable signal wherein said single-stranded DNA comprises a nucleotide sequence identical to at least one allele specific oligonucleotide sequence or differs by at least one nucleotide from the allele specific oligonucleotide sequence, said contact being under stringency conditions which permit differential hybridization between identical nucleotide sequences and sequences having at least one mismatch and then screening for the presence, absence or level of signal from the reporter molecule wherein the pattern of presence, absence or level of signal provides the identity of the genotype of the subject.

An important key feature of the present invention is the selection of genes or target nucleic acid sequences which differ in nucleotide sequence by at least one nucleotide between a healthy subject and a subject having genetic deafness or a predisposition for development of same. Suitable genes or nucleic acid target sequences include *inter alia connexion 26*, *pendrin*, mitochondrial 12S rRNA and *usherin*. However, the present invention extends to a range of other genes or target nucleotide sequences.

Another key feature of the present invention is the selection of stringency conditions required to induce differential hybridization capacity between identically complementary nucleotide sequences and those which differ by at least one nucleotide. Useful hybridization conditions for the practice of the present invention include 1-4 X SSC at 30-50°C for 15 min to 90 min followed by washing at 30-50°C in the following sequence:-

1-4 X SSC/0.05% - 0.4% SDS (1-5 min);
0.1-1 X SSC/0.05% - 0.4% SDS (2-10 min);
0.5 X -5 X SSC (0.5-3 min);
2-8 X SSC/0.05% (0.5-3 min); and
2-8 X SSC/0.05%-2% Tween (0.5-3 min).

These conditions may vary or may have to be modified for the particular genes or nucleic acid molecules being targeted. All such variations are encompassed by the present invention.

The immobilized oligonucleotides may be from about 5 to about 100 nucleotides in length although oligonucleotides outside this range are nevertheless still contemplated in accordance with the present invention. Particularly preferred oligonucleotides are from about 10 to about 50 nucleotides in length or from about 15 to about 30 nucleotides in length.

Accordingly, another aspect of the present invention provides a method for genotyping a human subject for a gene or target nucleotide sequence selected from *connexin 26, pendrin,* mitochondrial 12S rRNA and *usherin* wherein a mutation in one or more of the above genes or target nucleotide sequences is associated with genetic deafness or a propensity for genetic deafness to develop, said method comprising contacting an immobilized array of oligonucleotides which comprise a nucleotide sequence corresponding to a wild-type nucleotide sequence or a mutant nucleotide sequence or one or more of the above-mentioned genes or target nucleotide sequences with a single-

stranded DNA molecule labeled with a reporter molecule capable of providing an identifiable signal from said human subject or group of human subjects under stringency conditions which permit differential hybridization between identical nucleotide sequences relative to nucleotide sequences which differ by at least one nucleotide and recording the presence, absence or level of signal from the reporter molecule which indicates which oligonucleotide has an identical nucleotide sequence to a DNA sequence from a human subject.

The oligonucleotides immobilized to the array are referred to herein as "allele-specific oligonucleotides". The term "allele" is not to impart any limitation as to the function of the oligonucleotides. In essence, the nucleotide sequence of the oligonucleotide will encompass one or more nucleotides in a corresponding nucleotide sequence of a gene or target nucleic acid molecule, such as from *connexion 26, pendrin*, mitochondrial 12S rRNA or *usherin* but where at least one nucleotide in the gene or target nucleic acid molecule may differ between a healthy subject or a subject with genetic deafness or a propensity to develop same.

The oligonucleotides may comprise nucleotide sequences at the 5' or 3' ends to facilitate less folding of the oligonucleotides or to otherwise keep the sequence specific portion further away from the solid support.

In a particular embodiment, the present invention provides a set of one or more oligonucleotides having the sequence:-

$$[n]_x - A$$

wherein:

n is one or a range of different nucleotides;

x is the length of the nucleotide sequence [n]; and

A is a nucleotide sequence selected from SEQ ID NOs:33 to 64.

In one particular example, n is T and x is from about 5 to about 30 such as about 10. Specific examples of $[n]_x$ - A include the oligonucleotides defined by SEQ ID NOs:1 to 32. The $[n]_x$ portion may also be a chemical linker.

In one embodiment, the oligonucleotides comprise "wild-type" nucleotide sequences meaning that the nucleotide sequences correspond to the exact same sequence as in a gene or target from a healthy subject. In this case, if a single-stranded DNA sequence from one of the aforementioned genes or nucleic acid targets differs by at least one nucleotide from the oligonucleotide sequence, then, under the differential hybridization conditions employed, a DNA with non-identical nucleotide sequence will not substantially hybridize.

Similarly, the oligonucleotides could encompass nucleotide sequences which are derived from a mutated gene. In this case, only DNA from subjects with a mutated gene or target nucleic acid would substantially hybridize, The present invention encompasses both forms of arrays.

Accordingly, another aspect of the present invention contemplates a method for genotyping a human subject from a gene or nucleic acid target selected from *connexion 26*, *pendrin*, mitochondrial 12S rRNA and *usherin* whrein a mutation in one or more of these genes or targets is indicative of genetic deafness or a propensity to develop genetic deafness, said method incorporating a label directly or indirectly into genomic DNA amplified from the human subject to be tested using primers which flank a DNA sequence corresponding to a potential mutation in a gene or nucleic acid target listed above and contacting single-stranded labeled forms of the amplified DNA with an immobilized oligonucleotide selected from SEQ ID NO:1 to SEQ ID NO:64 under stringency conditions such that substantially only identically complementary DNA from the subject is capable of hybridizing to the corresponding immobilized oligonucleotide and screening for hybridization by measuring a signal or level of signal from the label.

The nucleotide sequence of the target nucleotide sequence may be modified up- or down-stream of a mutation to be detected or groups of mutations to be detected. This may be useful to interrupt a particular sequence of nucleotides to improve hybridization sensitivity. For example, mismatched primers may be used to introduce a mismatch within a sequence of G residues. This may be useful, for example, in relation to the target DNA sequence which hybridizes to a 35ΔG mutation in *connexion 26*. SEQ ID NOs:1 to 4, for example, include a sequence of six Gs. This sequence is disruptable by a non-G nucleotide. This is proposed to reduce oligonucleotide bending and improve hybriziation efficiency or sensitivity.

This approach applies to all the oligonucleotides of the present invention.

Preferably, the stringency conditions comprise 1-4 X SSC at 30-50°C for 15 min to 90 min followed by washing at 30-50°C in the following sequence:-

1-4 X SSC/0.05% - 0.4% SDS (1-5 min);
0.1-2 X SSC/0.05% - 0.4% SDS (2-10 min);
0.5 X -5 X SSC (0.5-3 min);
2-8 X SSC/0.05% (0.5-3 min); and
2-8 X SSC/0.05%-2% Tween (0.5-3 min).

Consequently, another aspect of the present invention is directed to a method for genotyping a human subject from a gene or nucleic acid target selected from *connexion 26, pendrin*, mitochondrial 12S rRNA and *usherin* wherein a mutation in one or more of these genes or targets is indicative of genetic deafness or a propensity to develop genetic deafness, said method incorporating a label into genomic DNA amplified from the human subject to be tested using primers which flank a DNA sequence corresponding to a potential mutation in a gene or nucleic acid target listed above and contacting single-stranded labeled forms of the amplified DNA with an immobilized oligonucleotide selected from SEQ ID NO:1 to SEQ ID NO:32 under stringency conditions of 1-4 X SSC at 30-50°C for 15 min to 90 min followed by washing at 30-50°C in the following

sequence:-

1-4 X SSC/0.05% - 0.4% SDS (1-5 min);
0.1-3 X SSC/0.05% - 0.4% SDS (2-10 min);
0.5 X -5 X SSC (0.5-3 min);
2-8 X SSC/0.05% (0.5-3 min); and
2-8 X SSC/0.05%-2% Tween (0.5-3 min);

such that substantially only identically complementary DNA from the subject is capable of hybridizing to the corresponding immobilized oligonucleotide and screening for hybridization by measuring a signal or level of signal from the label.

Any of a number of labels may be incorporated into the amplified test DNA. Fluorescent labels and fluorophores are particularly useful.

In one embodiment, a few cycles (e.g. 1 or 2 or 3 or 4 or 5) PCR is conducted using pairs of primers, one or both of which are generally labeled with the same or a different reporter molecule capable of giving a distinguishable signal. The use of fluorophores is particularly useful in the practice of the present invention. Examples of suitable fluorophores may be selected from the list given in Table 2. Other labels include luminescence and phosphorescence as well as infrared dyes. These dyes or fluorophores may also be used as reporter molecules for antibodies.

TABLE 2 List of suitable fluorophores

Probe	Ex[1] (nm)	Em[2] (nm)
Hydroxycoumarin	325	386
Aminocoumarin	350	455
Methoxycoumarin	360	410
Cascade Blue	375; 400	423
Lucifer Yellow	425	528
NBD	466	539
R-Phycoerythrin (PE)	480; 565	578
PE-Cy5 conjugates	480; 565; 650	670

Probe	Ex[1] (nm)	Em[2] (nm)
PE-Cy7 conjugates	480; 565; 743	767
APC-Cy7 conjugates	650; 755	767
Red 613	480; 565	613
Fluorescein	495	519
FluorX	494	520
BODIPY-FL	503	512
TRITC	547	574
X-Rhodamine	570	576
Lissamine Rhodamine B	570	590
PerCP	490	675
Texas Red	589	615
Allophycocyanin (APC)	650	660
TruRed	490, 675	695
Alexa Fluor 350	346	445
Alexa Fluor 430	430	545
Alexa Fluor 488	494	517
Alexa Fluor 532	530	555
Alexa Fluor 546	556	573
Alexa Fluor 555	556	573
Alexa Fluor 568	578	603
Alexa Fluor 594	590	617
Alexa Fluor 633	621	639
Alexa Fluor 647	650	688
Alexa Fluor 660	663	690
Alexa Fluor 680	679	702
Alexa Fluor 700	696	719
Alexa Fluor 750	752	779
Cy2	489	506
Cy3	(512); 550	570; (615)
Cy3,5	581	596; (640)
Cy5	(625); 650	670
Cy5,5	675	694
Cy7	743	767
Hoeschst 33342	343	483
DAPI	345	455
Hoechst 33258	345	478
SYTOX Blue	431	480
Chromomycin A3	445	575
Mithramycin	445	575
YOYO-1	491	509
SYTOX Green	504	523
SYTOX Orange	547	570
Ethidium Bormide	493	620
7-AAD	546	647

Probe	Ex[1] (nm)	Em[2] (nm)
Acridine Orange	503	530/640
TOTO-1, TO-PRO-1	509	533
Thiazole Orange	510	530
Propidium Iodide (PI)	536	617
TOTO-3, TO-PRO-3	642	661
LDS 751	543; *590*	712; *607*
Y66F	360	508
Y66H	360	442
EBFP	380	440
Wild-type	396, 475	50, 503
GFPuv	385	508
ECFP	434	477
Y66W	436	485
S65A	471	504
S65C	479	507
S65L	484	510
S65T	488	511
EGFP	489	508
EYFP	514	527
DsRed	558	583
Monochlorobimane	380	461
Calcein	496	517

1 Ex: Peak excitation wavelength (nm)

2 Em: Peak emission wavelength (nm)

Any suitable method of analyzing fluorescence emission is encompassed by the present invention. In this regard, the invention contemplates techniques including but not restricted to 2-photon and 3-photon time resolved fluorescence spectroscopy as, for example, disclosed by Lakowicz *et al., Biophys. J. 72:* 567, 1997, fluorescence lifetime imaging as, for example, disclosed by Eriksson *et al., Biophys. J. 2:* 64, 1993 and fluorescence resonance energy transfer as, for example, disclosed by Youvan *et al., Biotechnology et elia 3:* 1-18, 1997.

Luminescence and phosphorescence may result respectively from a suitable luminescent or phosphorescent label as is known in the art. Any optical means of identifying such label may be used in this regard.

Infrared radiation may result from a suitable infrared dye. Exemplary infrared dyes that may be employed in the invention include but are not limited to those disclosed in Lewis *et al., Dyes Pigm. 42(2):* 197, 1999, Tawa *et al., Mater. Res. Soc. Symp. Proc. 488* [Electrical, Optical and Magnetic Properties of Organic Solid-State Materials IV], 885-890, Daneshvar *et al., J. Immunol. Methods 226(1-2):* 119-128, 1999, Rapaport *et al., Appl. Phys. Lett. 74(3):* 329-331, 1999 and Durig *et al., J. Raman Spectrosc. 24(5):* 281-285, 1993. Any suitable infrared spectroscopic method may be employed to interrogate the infrared dye. For instance, fourier transform infrared spectroscopy as, for example, described by Rahman *et al., J. Org. Chem. 63:* 6196, 1998 may be used in this regard.

Suitably, electromagnetic scattering may result from diffraction, reflection, polarization or refraction of the incident electromagnetic radiation including light and X-rays. Such scattering can be used to quantitate the level of mRNA or level of protein.

Flow cytometry is particularly useful in analyzing fluorophore emission.

As is known in the art, flow cytometry is a high throughput technique which involves rapidly analyzing the physical and chemical characteristics of particles (e.g. labeled DNA) as they pass through the path of one or more laser beams while suspended in a fluid stream. As each particle intercepts the laser beam, the scattered light and fluorescent light emitted by each cell or particle is detected and recorded using any suitable tracking algorithm as, for example, described hereunder.

A modern flow cytometer is able to perform these tasks up to 100,000 cells/particles s^{-1}. Through the use of an optical array of filters and dichroic mirrors, different wavelengths of fluorescent light can be separated and simultaneously detected. In addition, a number of lasers with different excitation wavelengths may be used. Hence, a variety of fluorophores can be used to target and examine, for example, different immune effectors within a sample or immune effectors from multiple subjects.

Suitable flow cytometers which may be used in the methods of the present invention include those which measure five to nine optical parameters (see Table 3) using a single excitation laser, commonly an argon ion air-cooled laser operating at 15 mW on its 488 nm spectral line. More advanced flow cytometers are capable of using multiple excitation lasers such as a HeNe laser (633 nm) or a HeCd laser (325 nm) in addition to the argon ion laser (488 or 514 nm).

TABLE 3 Exemplary optical parameters which may be measured by a flow cytometer.

Parameter	Acronym	Detection angle form incident laser beam	Wavelength (nm)
Forward scattered light	FS	2-5°	488*
Side scattered light	SS	90°	488*
"Green" fluorescence	FL1	90°	510-540†
"Yellow" fluorescence	FL2	90°	560-580†
"Red" fluorescence	FL3	90°	>650#

* using a 488 nm excitation laser

† width of bandpass filter

\# longpass filter

For example, Biggs *et al., Cytometry 36:* 36-45, 1999 have constructed an 11-parameter flow cytometer using three excitation lasers and have demonstrated the use of nine distinguishable fluorophores in addition to forward and side scatter measurements for purposes of immunophenotyping (i.e. classifying) particles. The maximum number of parameters commercially available currently is 17: forward scatter, side scatter and three excitation lasers each with five fluorescence detectors. Whether all of the parameters can be adequately used depends heavily on the extinction coefficients, quantum yields and amount of spectral overlap between all fluorophores (Malemed *et al., "Flow cytometry and sorting"*, 2nd Ed., New York, Wiley-Liss, 1990). However, it will be understood that the present invention is not restricted to any particular flow cytometer or any particular set of parameters. In this regard, the invention also contemplates use in place of a conventional flow cytometer, a microfabricated flow cytometer as, for example, disclosed by Fu *et al.*,

Nature Biotechnology 17: 1109-1111, 1999.

Electrotides may also be used as a detection system. Such a system relies on complementary binding of RNA or DNA to assemble an electronic circuit which thereby creates a detectable electronic signal. One particularly useful system is eSensor (trade mark; Motorola) which is well described at http://www.motorola.com/lifesciences/esensor/tech_overview.htm1.

The signal produced following hybridization provides a genotype index (GI).

The GI is calculated by the algorithm:-

$$GI = \frac{SV_N}{SV_N + SV_M}$$

wherein:

SV_N is the normal spot value; and
SV_M is the mutant spot value.

Generally, a background subtracted median pixel intensity is used as the spot value.

Accordingly, a method for genotyping a human subject from a gene or nucleic acid target selected from *connexion 26, pendrin,* mitochondrial 12S rRNA and *usherin* wherein a mutation in one or more of these genes or targets is indicative of genetic deafness or a propensity to develop genetic deafness, said method incorporating a label into genomic DNA amplified from the human subject to be tested using primers which flank a DNA sequence corresponding to a potential mutation in a gene or nucleic acid target listed above and contacting single-stranded labeled forms of the amplified DNA with an immobilized oligonucleotide selected from SEQ ID NO:1 to SEQ ID NO:32 under stringency conditions of 1-4 X SSC at 30-50°C for 15 min to 90 min followed by washing at 30-50°C

in the following sequence:-

1-4 X SSC/0.05% - 0.4% SDS (1-5 min);
0.1-4 X SSC/0.05% - 0.4% SDS (2-10 min);
0.5 X -5 X SSC (0.5-3 min);
2-8 X SSC/0.05% (0.5-3 min); and
2-8 X SSC/0.05%-2% Tween (0.5-3 min);

such that substantially only identically complementary DNA from the subject is capable of hybridizing to the corresponding immobilized oligonucleotide and screening for hybridization by measuring a signal or level of signal from the label, wherein a GI value is determined by the algorithm:-

$$GI = \frac{SV_N}{SV_N + SV_M}$$

wherein:

SV_N is the normal spot value; and
SV_M is the mutant spot value;

such that:

if $0.8 < GI < 1.0$, then the genotype is N/N;
if $0.65 < GI < 0.5$, then the genotype is N/M; and
if $0.0 < GI < 0.2$, then the genotype is M/M;

wherein:

N is a normal allele; and
M is a mutant allele.

The present invention further contemplates an array of oligonucleotides selected fromt two or more of SEQ ID NOs:1 to 32 for use in a differential hybridization assay of DNA from a subject being tested for genetic deafness or a propensity for development of genetic deafness.

This aspect of the present invention provides a kit for use in screening subjects for the presence of genes or nucleic acid molecules such as mitochondrial 12S rRNA whicih are either "mutated" or "normal" (i.e. wild-type). A mutant gene or target is proposed to be associated with genetic deafness or a predisposition for developing genetic deafness. A "normal" gene or target is from a subject without genetic deafness.

The present method is also useful in designing therapeutic protocols for treating genetic deafness. A therapeutic protocol includes medical intervention as well as behavioral changes required by a subject who is likely to become deaf.

The present invention is further described by the following non-limiting Examples.

EXAMPLE 1

DNA preparation

1. Amplify patient DNA in three PCR reactions containing primer mixes 1, 2 and 3 (Table 35) according to Table 4:

TABLE 4 PCR reaction mixtures

VOLUME	CONTENT
x µl[1]	(50 ng) patient DNA
2.5 µl	10X Taq buffer
2.5 µl	10X primer mix
2.5 µl	10X nucleotide labeling mix
0.5 µl	Taq polymerase
y µl[2]	dH_2O
25 µl	

[1] volume required to provide 50 ng of DNA

[2] volume required to make up to 25 µl

PCR is one cycle of denaturation for 5 min at 94° followed by 40 cycles of denaturation for 30 s at 94°C, annealing for 30 s at 58°C and extension for 30 s at 72°C, followed by a final extension step for 5 min at 72°C.

2. Take 5 µl of each reaction for gel analysis (optional), pool the remaining DNA into one tube and purify on a Qiagen MinElute column according to the manufacturer's instructions. Elute in 12 µl 10 mM Tris-Cl pH 7.5.

3. Add 3 µl 5X T7 gene 6 exonuclease buffer and 0.5 µl T7 gene 6 exonuclease. Incubate 20 min at 37°C, then heat inactivate at 90°C for 10 min.

4. Touch spin to collect condensate and store at -20°C until use.

TABLE 5 Primer mixers[1]

Primer Mix 1	286 bp, 137 bp
P1	TCTTTTCCAGAGCAAACCGC [SEQ ID NO:65]
P3-thio	GsAsCsAsCsGAAGATCAGCTGCAG [SEQ ID NO:66]
h.48	CGTCACCCTCCTCAAGTATACTTC [SEQ ID NO:67]
h.6-thio	GsCsTsTsTsGTGTTAAGCTACACTCTGG [SEQ ID NO:68]
Primer Mix 2	248 bp, 217 bp, 139 bp
PDS6F	GGTTTCTATCTCAGGCAAACAT [SEQ ID NO:69]
PDS6R-thio	AsTsTsGsTsTTCTGGAATGAACAGTGACC [SEQ ID NO:70]
PDS8F2	TTCAGACGATAATTGCTACTG [SEQ ID NO:71]
PDS8R-thio	GsAsCsTsGsACTTACTGACTTAATG [SEQ ID NO:72]
PDS10F	GTAGGATCGTTGTCATCCAG [SEQ ID NO:73]
PDS10R-thio	CsGsAsGsCsCTTCCTCTGTTGC [SEQ ID NO:74]
Primer Mix 3	311 bp, 159 bp
P4	CTGCAGCTGATCTTCGTGTC [SEQ ID NO:75]
P7-thio	AsCsAsAsAsGCAGTCCACAGTGTT [SEQ ID NO:76]
USH2AF	ATGTGAGCCCTGCCAGTGTA [SEQ ID NO:77]
USH2AR-thio	TsCsAsCsAsGGCCTTACAATTGGTG [SEQ ID NO:78]

s phosphorothioate bond

1 10X primer mixes are 4 μM each primer.

Notes

- 10X nucleotide labeling mix = 2 mM dATP, dCTP, dGTP, 1.5 mM dTTP and 0.5 mM biotin-dUTP.
- A hot start PCR protocol is preferred.

- T7 gene 6 exonuclease can be obtained from USB (Cat. No. 70025). It is used to prepare ss DNA by selectively digesting the strand that is not protected by a phosphorothioated primer.

EXAMPLE 2

Hybridization and labeling

1. Add 5 µl pooled ss PCR products to 5 µl hybridization buffer. Mix thoroughly.

2. Denature 5 min at 90°C.

3. Snap cool hybridization mix on ice.

4. Touch spin to collect condensate and pipette 10 µl hybridization mix onto a clean coverslip. Lower the measuring region of the chip onto the coverslip and let the hybridization mix spread to the edges of the coverslip.

5. Put the clip into a hybridization cassette containing 2X SSC in the humidification wells and incubate in a 45°C water bath for 30 min.

6. Wash chip at 45°C in the following sequence:

2X SSC/0.1% w/v SDS	3 min
0.5X SSC/0.1% w/v SDS	5 min
2X SSC	1 min
4X SSC/0.2% w/v Tween	1 min.

7. Let chip drain briefly but *do not allow to dry out*. Pipette 12 µl streptavidin-Cy5 diluted 1:250 in blocking solution onto a coverslip, avoiding bubbles. Lower the measuring region of the chip onto the coverslip and let the solution spread to the edges of the coverslip. Incubate in a damp chamber in the dark at RT for 30-60

min.

8. Wash the chip 2 X 3 min in 4X SSC/0.2% w/v Tween at 45°C.

9. Rinse the chip in 0.1X SSC at RT for 2 min.

10. Dry chip by centrifugation in a 50 mL Falcon tube at 500 rpm for 3 min and store in dark, dry place until scanning.

Notes

- Hybridization buffer = 5X SSPE/0.01% v/v Triton X100.
- Blocking solution = 4X SSC/0.2% w/v Tween20/5% w/v BSA.
- Streptavidin-Cy5 can be obtained from a number of suppliers (e.g. Amersham PA45001).

EXAMPLE 3

Scanning and analysis

1. Scan the chip in a standard microarray scanner using the red Cy5 channel (635 nm).

2. Quantitate spot intensities using the scanner software. At this time, visually inspect the array and exclude any "bad" spots (e.g. poor printing or hybridization, contamination by dust particle, etc.).

3. Import results into Excel. Using the background, subtract median pixel intensity as your Spot Value (SV), calculate the Genotype Index (GI) for each normal and mutant spot pair.

$$GI = SV_N / (SV_N + SV_M)$$

where SV_N = normal Spot Value and SV_M = mutant Spot Value

4. Average GI values for replicate spot pairs and use to call genotype for each mutation.

EXAMPLE 4

Attachment of oligonucleotides

Oligonucleotides are attached to the solid support by coupling *via* an epoxide group on the solid support. This is shown in Figure 1.

EXAMPLE 5

Microarray based genotyping

Figure 2 shows the principle of microarray genotyping. Oligonucleotides covering mutant or normal sequences are immobilized to a solid support using the coupling reaction described in Example 4. A single-stranded labeled DNA from a test substrate is then brought into contact using hybridization conditions which facilitate differential hybridization. A signal is then measured to ascertain binding or no binding.

EXAMPLE 6

Genotyping of connexin 26 35ΔG and M34T mutations

Figure 3 shows the results of the microarray assay. The genotypes N/N, 35ΔG/M34T 35ΔG/35ΔG and M34T/M34T are clearly discernible.

The intensity of the signal provides a means of calculating the GI.

The GI is calculated as follows:-

$$GI = \frac{SV_N}{SV_N + SV_M}$$

Figure 4 shows a graphical representation of the GI for the connexin 26 35ΔG and M34

mutations.

This experiment is repeated using a greater range of oligonucleotides. The results ar shown in Figure 5. A genotypic graph is shown in Figure 7.

EXAMPLE 7

Genotyping pendrin and 12S rRNA mutations

Figure 6 shows genotyping of *pendrin* and 12S rRNA mutations.

A genotypic summary is shown in Figure 7.

Figure 8 summarizes the results of applying the GI to deciding whether a subject is norma (N) homozygous, N heterozygous or a mutant (M) homozygous.

EXAMPLE 8

Potential interactions between deafness genes

Figure 9 shows the results of a potential interaction between deafness genes.

Those skilled in the art will appreciate that the invention described herein is susceptible t variations and modifications other than those specifically described. It is to be understoo that the invention includes all such variations and modifications. The invention als includes all of the steps, features, compositions and compounds referred to or indicated i this specification, individually or collectively, and any and all combinations of any two o more of said steps or features.

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Yoshinaga, *Otolaryngol Clin. North Am. 32(6):* 1089-1102, 1999.

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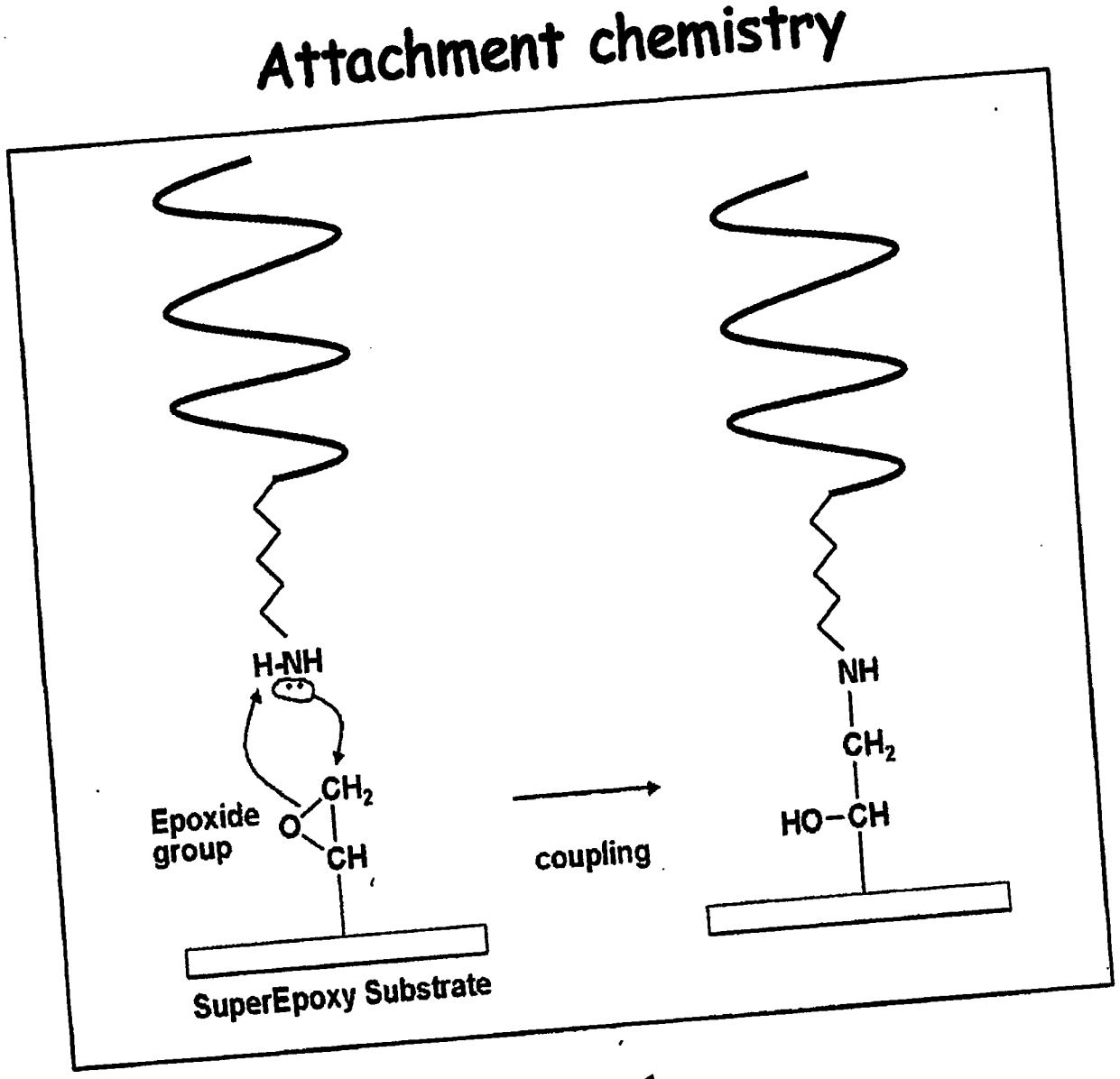

Attachment chemistry
H-NH
Epoxide group
O
CH2
CH
coupling
NH
CH2
HO–CH
SuperEpoxy Substrate

Figure 1

Microarray based genotyping

Allele specific
oligonucleotides (ASO)

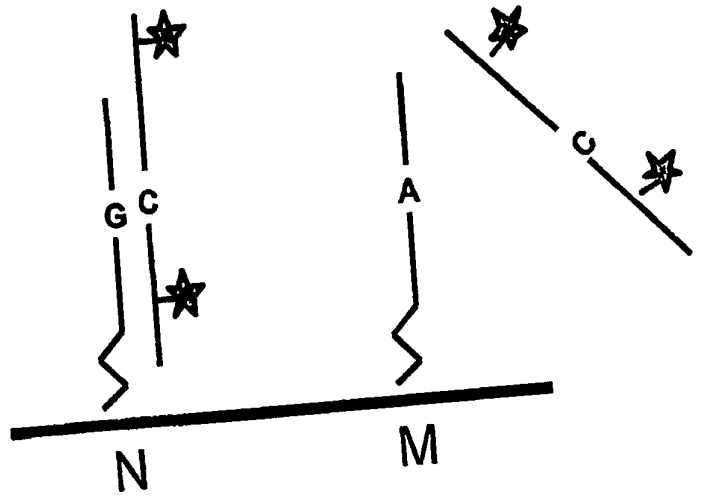


Figure 2

Step 1: Genotyping of connexin 26 35ΔG and M34T mutations (i)

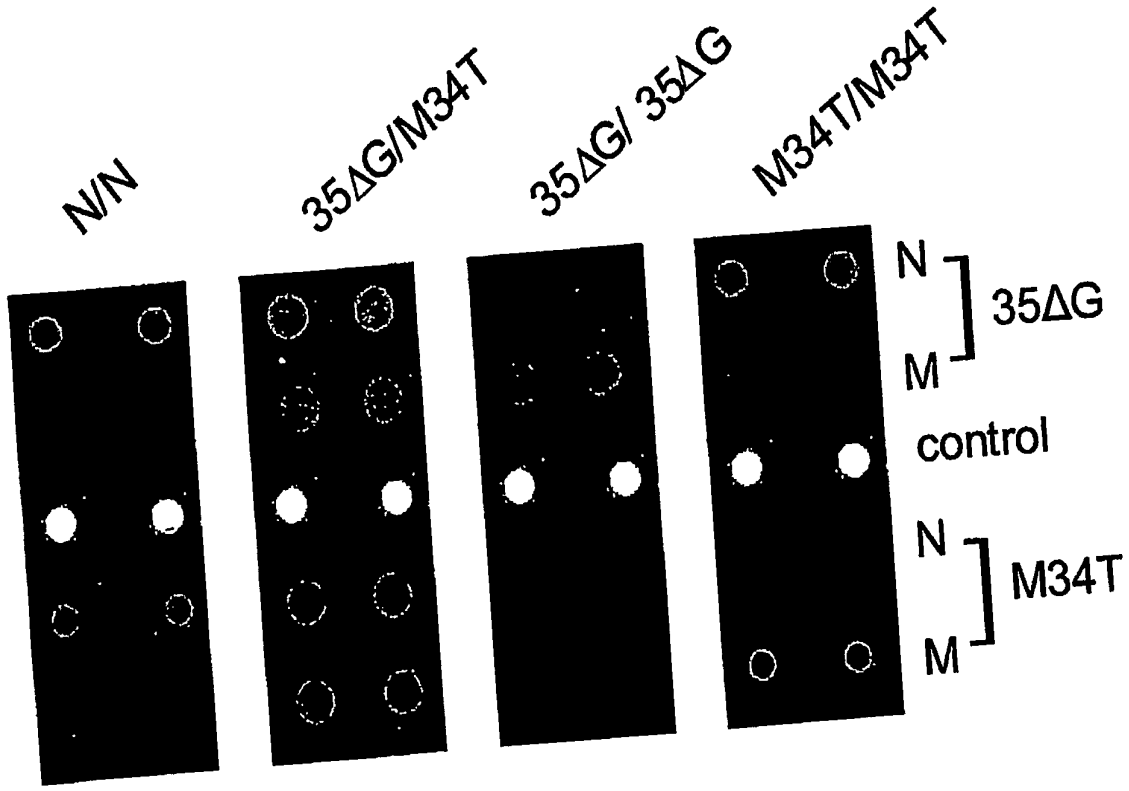


Figure 3

Step 1: Genotyping of connexin 26 35ΔG and M34T mutations (ii)

$$\text{Genotype Index (GI)} = \frac{SV_N}{(SV_N + SV_M)}$$

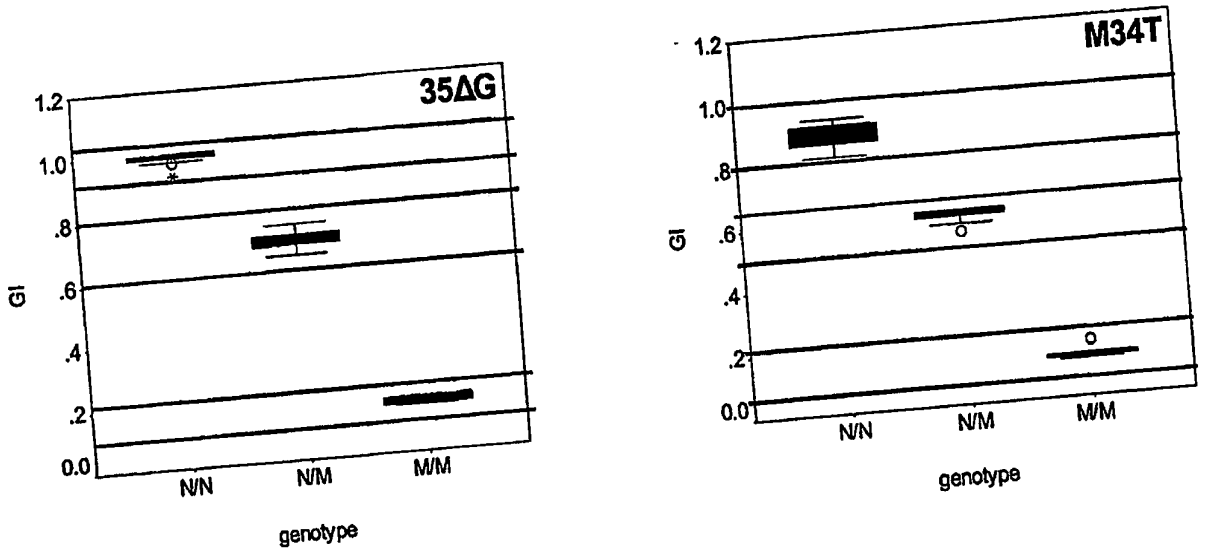


Figure 4

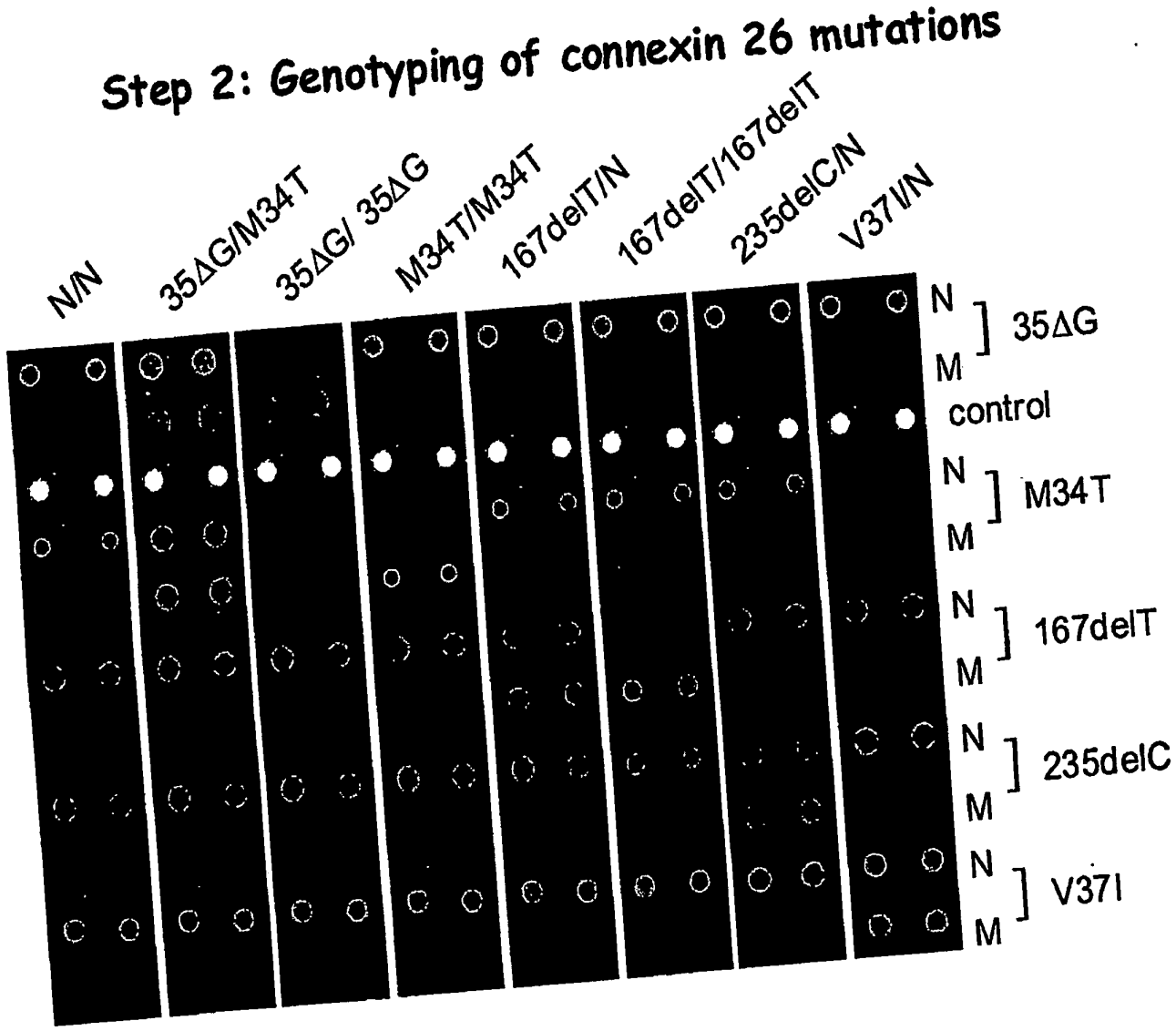

Step 2: Genotyping of connexin 26 mutations
N/N
35ΔG/M34T
35ΔG/ 35ΔG
M34T/M34T
167delT/N
167delT/167delT
235delC/N
V37I/N
N
M
35ΔG
control
M34T
167delT
235delC
V37I

Figure 5

Step 2: Genotyping of pendrin and 12S rRNA mutations

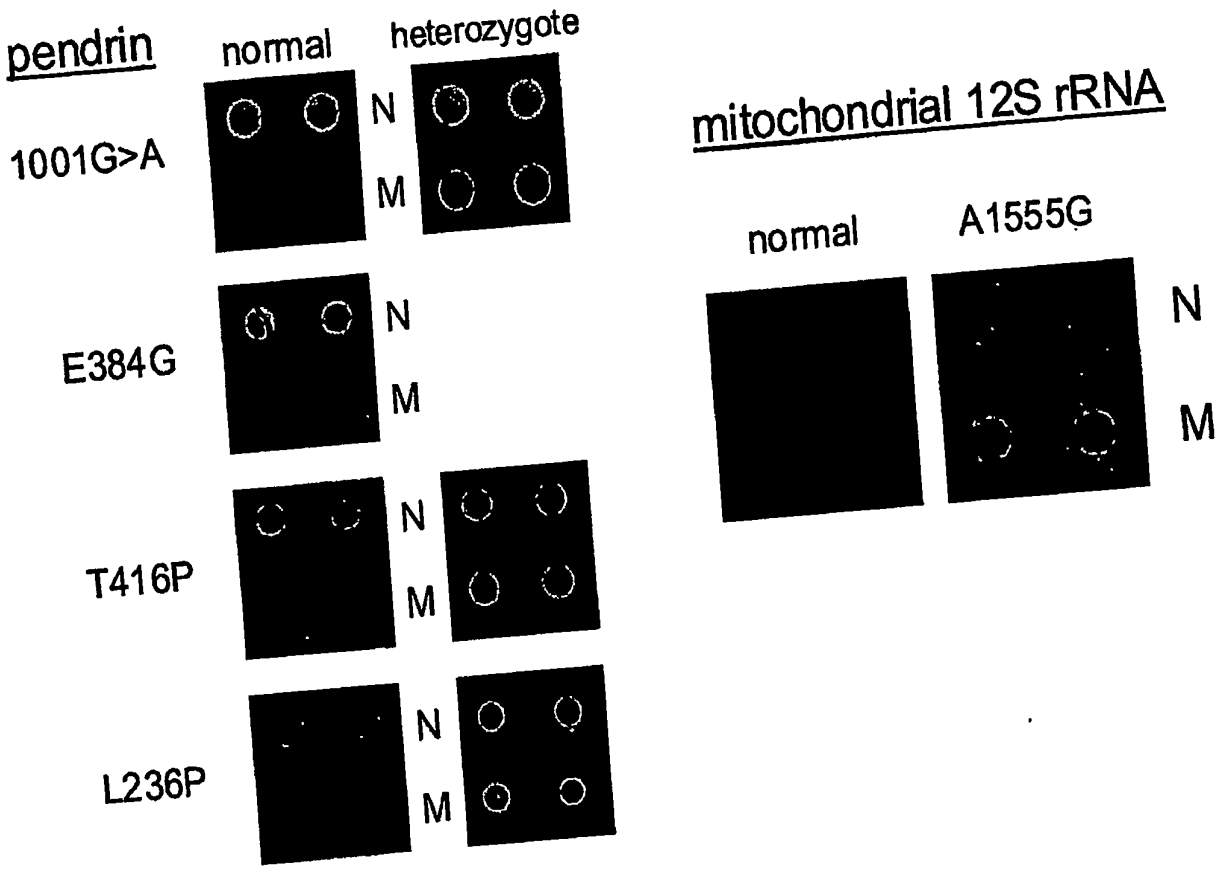


Figure 6

Genotyping summary

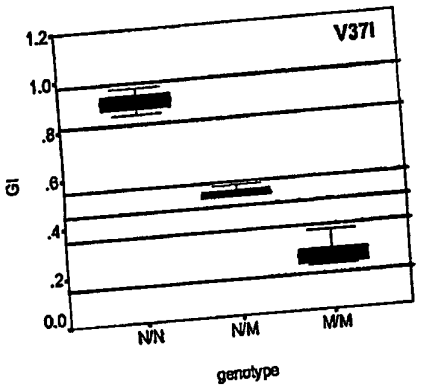


Figure 7A

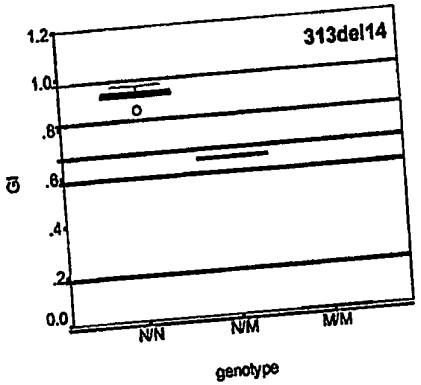


Figure 7C

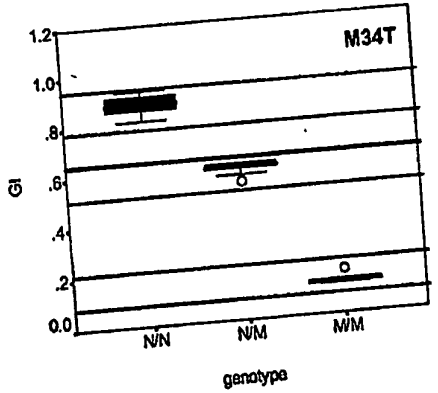


Figure 7E

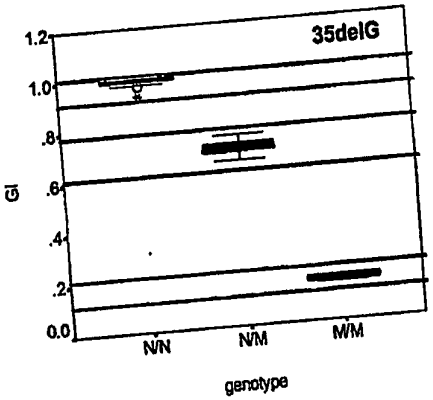


Figure 7B

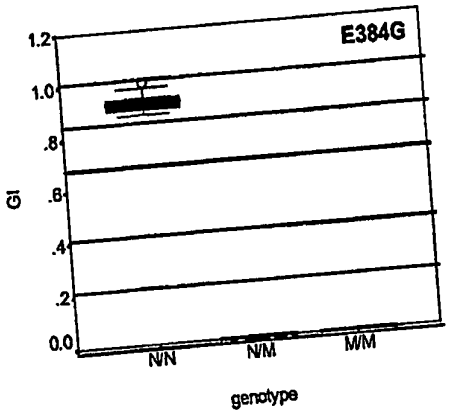


Figure 7D

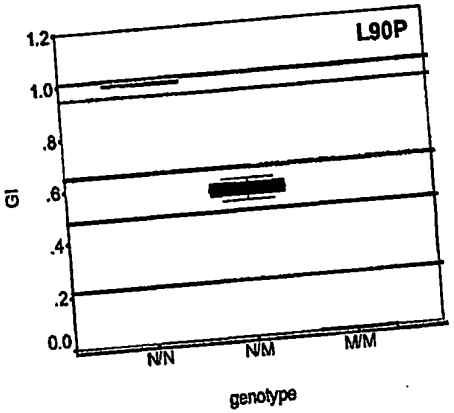


Figure 7F

Genotyping summary

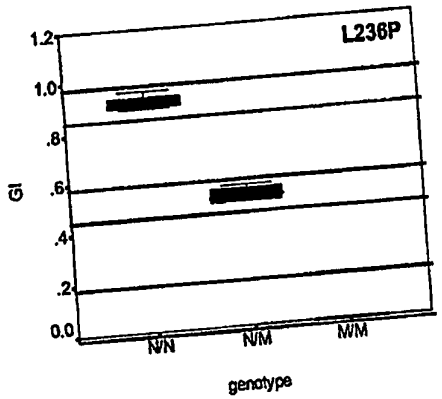


Figure 7G

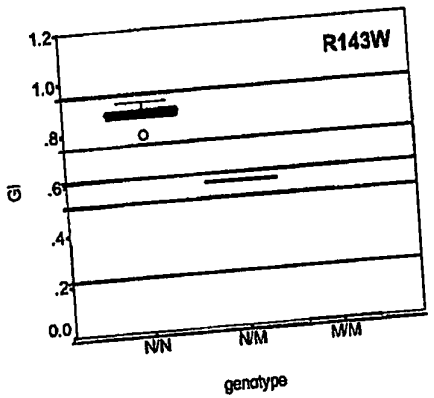


Figure 7I

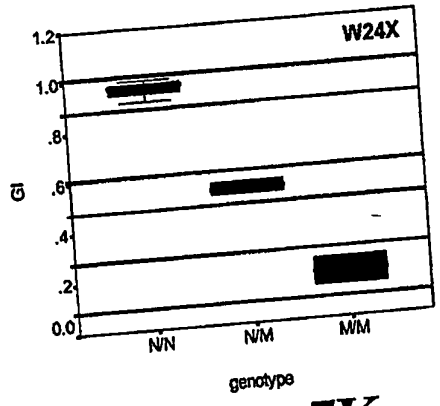


Figure 7K

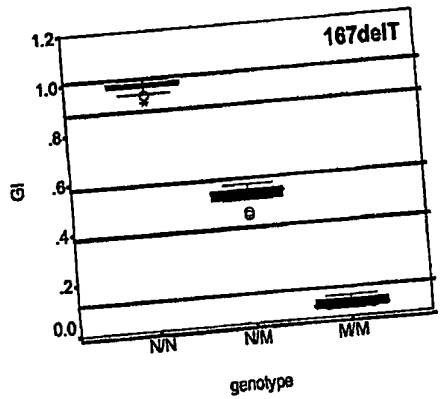


Figure 7H

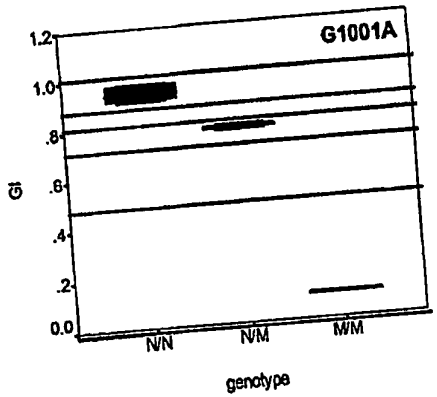


Figure 7J

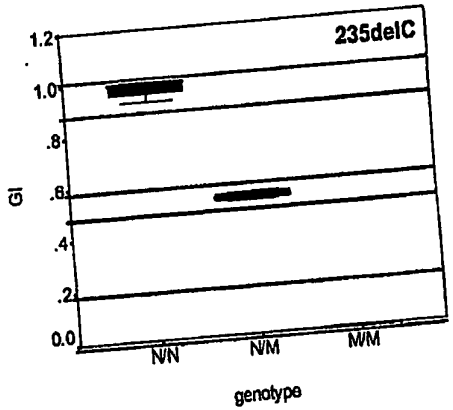


Figure 7L

Genotyping summary

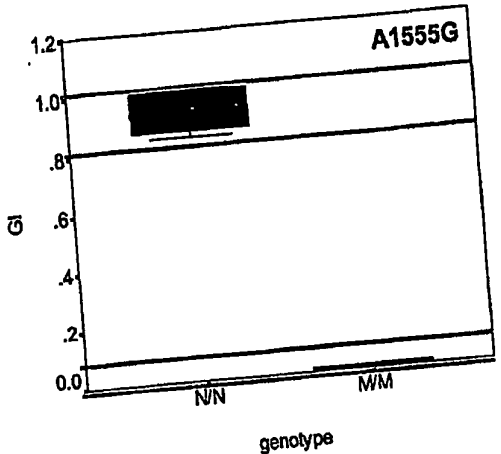


Figure 7M

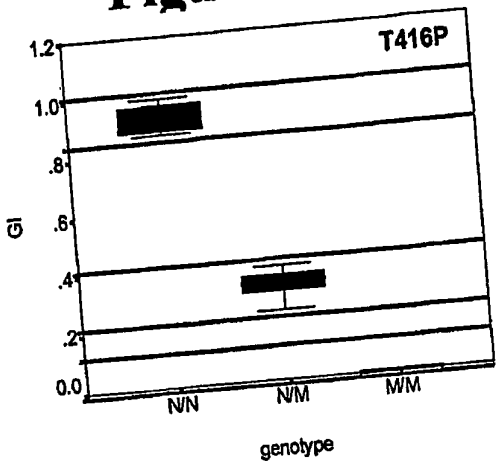
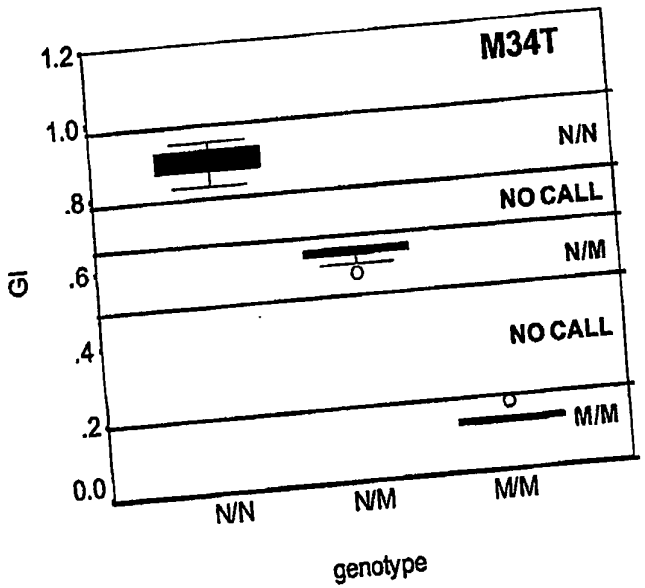


Figure 7N

Genotype calling algorithm



If 0.8<GI<1.0 then call = N/N

If 0.65<GI<0.8 then call = NO CALL

If 0.65<GI<0.5 then call = N/M

If 0.2<GI<0. 5 then call = NO CALL

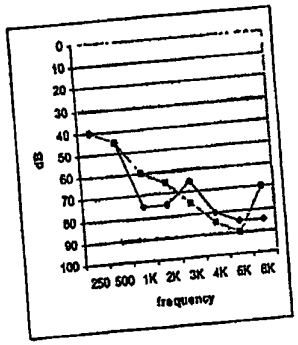
If 0.0<GI<0.2 then call = M/M

If $SI_N+SI_M<100$ then call = NO CALL

Figure 8

Interactions between deafness genes?

- severe hearing loss
- progressive
- age of onset 10 years



Mutation	GI	Call
connexin 26		
35delG	0.992806	N/N
W24X	0.93836	N/N
M34T	0.619185	**N/M**
V37I	0.902981	N/N
167delT	0.999151	N/N
235delC	0.997346	N/N
L90P	0.992122	N/N
R143W	0.862635	N/N
313del14	0.932165	N/N

Mutation	GI	Call
pendrin		
L236P	0.935414	N/N
G1001A	0.906897	N/N
E384G	0.941176	N/N
T416P	0.969543	N/N
12S rRNA		
A1555G	0.007764	**M/M**
usherin		
2299delG	0.995937	N/N

Figure 9